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Engrailed controls the organization of the ventral nerve cord through *frazzled* regulation

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Abstract

In *Drosophila*, the ventral nerve cord (VNC) architecture is built from neuroblasts that are specified during embryonic development, mainly by transcription factors. Here we show that Engrailed, a homeodomain transcription factor known to be involved in the establishment of neuroblast identity, is also directly implicated in the regulation of axonal guidance cues. Posterior commissures (PC) are missing in *engrailed* mutant embryos, and axonal pathfinding defects are observed when Engrailed is ectopically expressed at early stages, prior to neuronal specification. We also show that *frazzled*, *enabled*, and *trio*, all of which are potential direct targets of Engrailed and are involved in axonal navigation, interact genetically with *engrailed* to form posterior commissures in the developing VNC. The regulation of *frazzled* expression in *engrailed*-expressing neuroblasts contributes significantly to the formation of the posterior commissures by acting on axon growth. Finally, we identified a small genomic fragment within intron 1 of *frazzled* that can mediate activation by Engrailed *in vivo* when fused to a GFP reporter. These results indicate that Engrailed's function during the segregation of the neuroblasts is crucial for regulating different actors that are later involved in axon guidance.

Keywords: Engrailed; Frazzled; CNS development; *Drosophila*; Axon guidance

Introduction

The establishment of the ventral nerve cord (VNC) architecture is a complex and tightly regulated process. In *Drosophila* embryos, neuroblasts (NBs) acquire their unique identities through a combination of positional information along the antero-posterior (A/P) and dorso-ventral (D/V) axes (Dittrich et al., 1997; Bhat, 1999; Skeath, 1999; Skeath and Thor, 2003; Maurange and Gould, 2005). After delamination from the neuroectoderm, each NB divides asymmetrically to give rise to an NB and a ganglion mother cell (GMC). GMCs then divide once to produce either two neurons or one neuron and one glial cell (Bhat and Schedl, 1997). Between embryonic stages 9 and 13, NBs undergo several waves of mitosis and acquire their identities, whereas neuronal differentiation does not begin until stage 11, following the first wave of NB delamination.

The *Drosophila* embryonic VNC is composed of two longitudinal axonal tracts, with two commissures in each segment: the anterior and posterior commissures (ACs and PCs, respectively). During embryonic development, axonal projections must find their way among different cues that help determine whether they cross the midline through the commissures or continue along the longitudinal connectives (Tessier-Lavigne and Goodman, 1996). Such axon navigation decisions are tightly controlled, in part by cell surface-specific receptors that recognize chemoattractant or chemorepellent cues (Kolodziej et al., 1996; Mitchell et al., 1996; Araujo and Tear, 2003; Kinrade et al., 2001; Bhat, 2005). Although several of the factors that control midline crossing have been identified over the past few years, relatively little is understood about the transcriptional regulation of the genes encoding the guidance machinery. Indeed, whereas a number of transcription factors, including the segment polarity genes, have been found to regulate neuroblast formation and identity specification (Dittrich et al., 1997; Bhat, 1999), very few transcription factors have been shown to control pathfinding through the regulation of specific axon guidance receptors (Crowner et al., 2002; Fujioka et al., 2003; McDonald et al., 2003; Labrador et al., 2005).

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The homeodomain transcription factor Engrailed is involved in epidermal patterning, giving a posterior identity to cells in each segment (Lawrence and Struhl, 1996). *engrailed* is also expressed in NBs and in a subset of GMCs and neurons (Brower, 1984; Patel et al., 1989). During NB segregation, Engrailed is involved in the determination of NBs in rows 6 and 7 and of NBs 1–2 in row 1 (Bhat and Schedl, 1997; Lundell et al., 1996).

In the present study, we have aimed to understand the role of *engrailed* function in the organization of the ventral nerve cord. This was first carried out by testing the effects of *engrailed* loss- or gain-of-function on the VNC architecture. We found that Engrailed is involved in the decision over where axons cross the midline and in the choice of commissures. In particular, our analysis shows that *engrailed*-expressing cells are involved in the formation of the posterior commissures (PCs). We also present evidence showing that Engrailed acts strongly on axonal navigation during the segregation of the neuroblasts, but only has a weak influence over this process once neuronal cell fate is established.

Next, we analyzed the role of *engrailed* function on scaffold formation in the VNC by studying direct target genes that are involved in this process. In a previous study, we had identified 203 potential direct Engrailed target genes using a chromatin immunoprecipitation (ChIP) strategy (Solano et al., 2003). Several of these genes, such as *frazzled*, *trio* and *enabled*, were already known to be involved in axonal pathfinding (Forsthoefel et al., 2005). Here, we have identified strong genetic interactions between *engrailed* and these three genes. We particularly focused on *frazzled*, which encodes a Netrin receptor and plays an important role in axon guidance (Kolodziej et al., 1996). Frazzled is a *Drosophila* member of the Deleted in Colorectal Cancer (DCC) immunoglobulin subfamily, and is expressed in axons during central nervous system development (Kolodziej et al., 1996). This receptor is expressed at high levels on commissural axons, and captures Netrin in order to direct axon growth through the midline (Hiramoto et al., 2000). We show here that Engrailed and its direct target *frazzled* are necessary to insure the growth of axons that project through PCs. Interestingly, we have found that Engrailed mainly acts on *frazzled* expression during the segregation of the neuroblasts. Together, these results show that *engrailed* plays a crucial role in the organization of the ventral nerve cord, acting on axon guidance decisions. In particular, we have been able to show that, apart from its known role in the specification of neuroblast identity (Patel et al., 1989; Brower, 1984; Bhat and Schedl, 1997; Lundell et al., 1996), Engrailed is also involved in the regulation of molecules such as the Netrin-receptor Frazzled that directly govern axon growth (Forsthoefel et al., 2005).

Materials and methods

Drosophila strains

All crosses were raised at 25°C. The *engrailed* mutant strains used in this study were described in Gustavson et al. (1996). en^{E} corresponds to a deficiency that deletes parts of *engrailed* (*en*) and of the *en*-related gene, *invected* (*inv*), whereas *Def(2R)SFX31* (en^{X31}) completely deletes *en* and *inv*. en^{B86} and inv^{30} correspond to amorphic mutations. The *frazzled* mutants (fra^{1}, fra^{2}, fra^{3} and fra^{4}) were provided by P. Kolodziej. *enabled* (*ena*) mutants (ena^{02029} and ena^{GC5}) and *trio* mutants ($trio^{6A}$ and $trio^{KG06642}$) were provided by the Bloomington stock center. For genetic interaction analysis, all the lines tested were balanced with a chromosome marked with *krüppel*-GFP in order to unambiguously select transheterozygous mutants (Casso et al., 1999). Stage 13–16 embryos were filleted to get flat preparations, according to Benveniste et al. (1998), and scored for VNC defects. Heterozygous *tramtrack* mutant (ttk^{804}) flies (Fauvarque and Dura, 1993) were used to ectopically express En in salivary glands to perform immuno-FISH experiments on polytene chromosomes. The following transgenic stocks were used: UAS-Engrailed (Tabata et al., 1995), UAS-HA-VP16-En (Alexandre and Vincent, 2003), UAS-mCD8-GFP (Lee and Luo, 1999), UAS-Frazzled (Kolodziej et al., 1996), *engrailed*-Gal4 (Tabata et al., 1995), *MS1096*-Gal4 (Milan and Cohen, 2000), *elav*-Gal4 (Luo et al., 1994), *scabrous*-Gal4 (*sca*-Gal4) (Budnik et al., 1996), *daughterless*-Gal4 (*da*-Gal4) (Wodarz et al., 1995), Gal4-1407 (Luo et al., 1994), *paired*-Gal4 (*prd*-Gal4) (Alexandre and Vincent, 2003), and *eagle*-Gal4 (*eg*-Gal4) (Lundell and Hirsh, 1998).

The transgenic lines were obtained after cloning and injection of PCR-generated genomic fragments in a modified pWHT-*hsp70*-GFP vector into w^{1118}. Because Frazzled is a transmembrane receptor, we replaced GFP with mCD8-GFP, a transmembrane modified form of GFP from pBS-mCD8-GFP (Lee and Luo, 1999), in order to follow axons and cell membranes. Engrailed binding region within *frazzled* was amplified from genomic DNA using specific primers (2C5 up: GAAAGATCTCAATAAGCTGCTGC, 2C5 low: GCGGGATCCCGCTCCATCTCGAATCATTC), digested with *Bam*HI and *Bgl*II, and then inserted into pWHT-*hsp70*-mCD8-GFP as a monomer. This fragment corresponds to a previously described En-immunoprecipitated fragment (Solano et al., 2003), with an additional 50 bp on each side of the fragment that added 2 potential En binding sites. Three independent lines were tested and showed the same results.

RNA in situ hybridization and immunostaining

Immunostainings were performed on larvae as previously described (Solano et al., 2003). Fixation of embryos, and *in situ* hybridization using DIG-labelled antisense RNA probes were performed as previously described (Alexandre et al., 1996). For double staining in embryos, *in situ* hybridization was first performed using an alkaline phosphatase-based detection system (Roche), followed by incubation with a rabbit polyclonal anti-Engrailed antibody (dilution 1:300) overnight at 4°C and detection with HRP (Vector-Vectastain). Embryos were dehydrated and mounted in Canada Balsam for observation. For fluorescent immunostainings, either whole mount or flat dissected preparations of embryonic CNS (Benveniste et al., 1998) were performed as indicated, and the following primary antibodies were used: rabbit anti-Fra (1:1000) (Kolodziej et al., 1996), rabbit polyclonal anti-Engrailed antibody (1:300) (Solano et al., 2003), monoclonal anti-Engrailed antibody (1:150) (4F11, a generous gift of Nipam Patel), goat anti-HRP-Cy3-conjugated (1:100) (Jackson ImmunoResearch Laboratories), rabbit anti-GFP (1:1000) (Molecular Probes), and rabbit anti-SoxN (Cremazy et al., 2001). Cy2- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Images were taken at the facilities of the CRIC using a BioRad 1024 Laser Scanning Confocal microscopy.

Immuno-FISH on polytene chromosomes

Squashes were performed in a *tramtrack* mutant background (ttk^{804}), which allows *engrailed* expression in the salivary glands (Serrano et al., 1995). Immuno-FISH experiments were performed as described (Lavrov et al., 2004; Chanas et al., 2004). pWHT-*hsp70*-mCD8-GFP was labelled using the Bionick labelling system (Invitrogen), used as a probe, and detected using a fluorescein anti-biotin antibody (Vector) (1:200). Polyclonal anti-Engrailed antibodies (1:40), secondarily detected using a Cy3 anti-rabbit antibody (1:200), were used to identify Engrailed-binding sites. Chromosomal banding was detected with DAPI.

Results

Engrailed activity is important for axonal pathfinding during early embryonic stages

The architecture of the ventral nerve cord (VNC) can be visualized by HRP immunostaining. In stage 15 embryos, En is expressed in cells posterior to PCs (Fig. 1A). In order to visualize the projections of *en* expressing cells, we analyzed embryos carrying the *en*-Gal4 and UAS-mCD8-GFP transgenes, which together lead to the production of membrane-associated GFP protein in the *en* expression domains. We observed that most *en*-expressing neurons project axons through PCs (Figs. 1B–D). In homozygous *en* mutant embryos, the architecture of the VNC was highly disturbed (compare Figs. 1E–G with Fig. 1B). In amorphic en^{B86} mutants (Fig. 1E), or with deficiencies covering both *en* and the *en*-related gene *invected* (*inv*) (Figs. 1F, G), commissures were fused (arrow) or PCs were thinner than normal or missing (arrowhead) (Figs. 1E–G), suggesting that axons were either misrouted or absent. Longitudinal tracts were globally less affected than commissures in these mutants. These phenotypes suggest a role for *engrailed* (and *invected*) in the projection of axons through the midline, mostly in PCs.

Using the UAS/Gal4 system (Brand and Perrimon, 1993), we analyzed the effects of En gain-of-function on the VNC architecture. For this purpose, we used various Gal4 fly strains to drive En expression.

We first used the *prd*-Gal4 driver to ectopically express En in even segments, but not in odd segments, which thus served as an internal control. In *prd*-Gal4/UAS-mCD8-GFP embryos, GFP expression was already detectable in epidermal cells at stage 10, and was also present in NBs, as it co-localized with the NB-specific marker SoxNeuro (Cremazy et al., 2001) (data not shown). At stage 15, GFP was expressed in sensory neurons and in interneurons of even segments, as shown by HRP-immunostaining, (Fig. 2A) and was also expressed in glial cells, as detected by anti-Repo immunostaining (data not shown). GFP-positive neurons projected axons in longitudinal tracts, in PCs, and in ACs of even segments, and also projected a few axons in odd segments (data not shown). These results indicated that *prd*-Gal4 was suitable for ectopically expressing En in NBs and, later, in neuronal and glial cells.

Driving En expression with *prd*-GAL4 (Figs. 2A–C) led either to the fusion of ACs and PCs in even segments (Fig. 2B, arrowhead) or to the absence of these commissures (Fig. 2B, arrow). In the latter case, the longitudinal tracts appeared thicker (Figs. 2B, C). In *prd*-Gal4/UAS-En embryos, the number of cells was not affected where En was ectopically expressed in comparison to in normal odd segments (data not shown), showing that the number of neurons remains unchanged in a

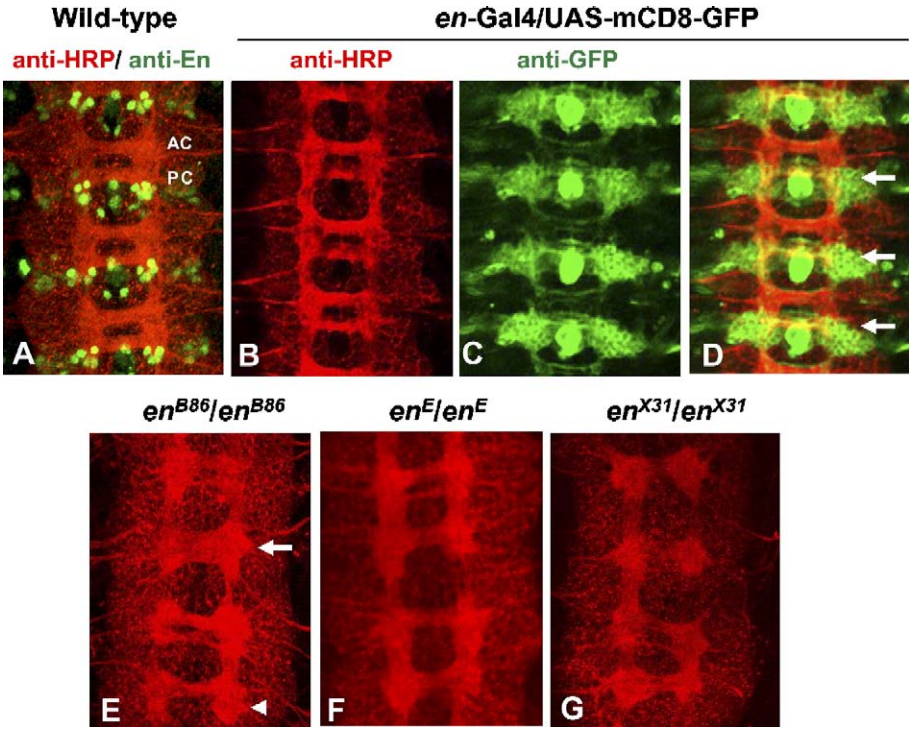

Fig. 1. Engrailed expression and *engrailed* mutant phenotype in stage 15 embryonic ventral nerve cords. Flat preparation of stage 15 embryos labelled with a Cy3-conjugated anti-HRP antibody to visualize axons (red) (A, B, D–G). (A) In wild-type, the VNC is organized into two longitudinal tracts on both sides of the midline, with two commissures per segment (AC and PC) crossing the midline. Engrailed is detected with a polyclonal anti-En antibody (green) and is expressed in neurons posterior to the PC. (B–D) VNC from *en*-Gal4/UAS-mCD8-GFP embryos. (B) VNC architecture visualized by anti-HRP-Cy3 immunostaining. (C) Membrane associated GFP labels axons emerging from En-expressing cells. (D) Overlay of panels B and C. Note that En-expressing neurons project through PCs (arrows). (E) en^{B86} homozygous embryo showing VNC defects with PCs fused (arrow) or absent (arrowhead). (F) en^{E} homozygous embryo showing a VNC disrupted with one commissure missing. (G) en^{X31} homozygous embryo showing a VNC highly perturbed with commissures fused or missing. Embryos are oriented anterior up. Pictures correspond to stacked confocal pictures.

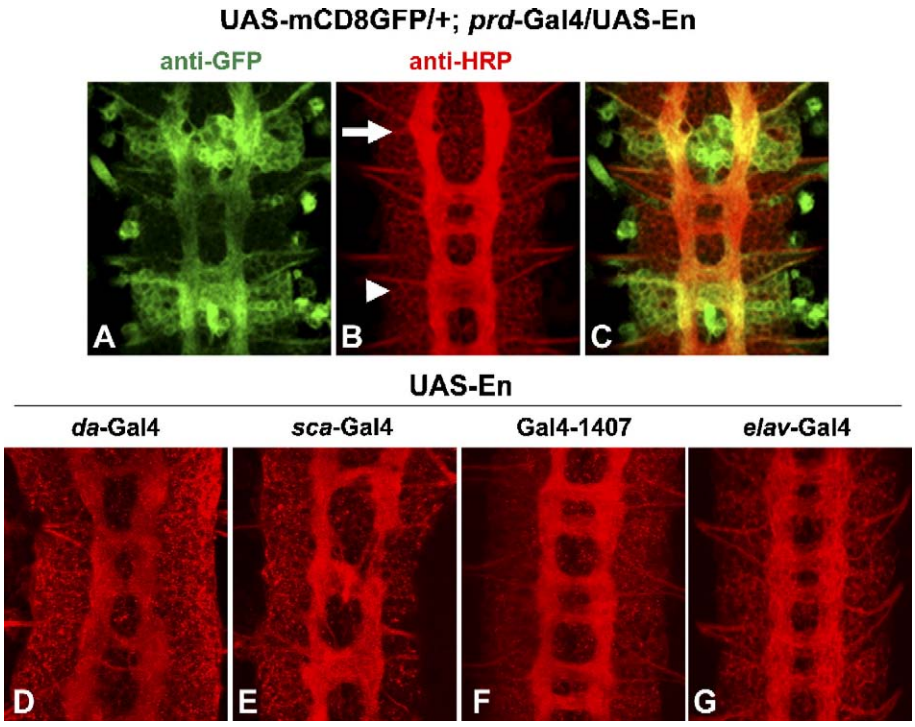

Fig. 2. Early ectopic Engrailed expression causes ventral nerve cord defects. (A–C) UAS-mCD8-GFP/+; *prd*-Gal4/UAS-En embryos. (A) GFP labelling with anti-GFP shows the cells that ectopically express En in even segments. (B) HRP labelling shows that commissural axons are missing (arrow) or fused (arrowhead). (C) Overlay of panels A and B shows that defects in the CNS appear in even segments. (D–G) HRP-Cy3 staining in: (D) *da*-Gal4/+; UAS-En/+ embryo, (E) *sca*-Gal4/+; UAS-En/+ embryo, (F) Gal4-1407/UAS-En and (G) *elav*-Gal4; UAS-En/+ (note that at 29°C, 1% of the embryos of this genotype present defects in VNC architecture) embryo. Embryos are dissected and oriented anterior up. Pictures correspond to stacked confocal pictures.

context of En overexpression. Altogether, these results indicated that the observed phenotypes were caused by axonal misrouting rather than missing axons, suggesting that Engrailed is involved in axonal navigation.

The use of *da*-Gal4, a ubiquitous early driver (Fig. 2D), and *sca*-Gal4, which is expressed in NBs and neurons (Fig. 2E), confirmed that ectopic expression of En can affect axonal pathfinding.

In order to better define the En-sensitive period for axonal pathfinding, we also tested pan-neuronal drivers such as Gal4-1407 (Fig. 2F) and *elav*-Gal4 (Fig. 2G). We did not observe, in comparison to wild-type (Fig. 1B), any major defects in the overall architecture with these drivers, or at most only low penetrance effects (less than 1%). This suggests that driving En expression in neurons does not strongly disturb VNC development.

These data show that the ectopic expression of En prior to neuronal specification can misroute axons, whereas En has no major effect when ectopically expressed in neurons only. Therefore, the action of En on axonal pathfinding occurs at early stages, before neuronal cell fate is established.

engrailed interacts genetically with several genes involved in axonal pathfinding

One way to understand how *engrailed* acts on axonal pathfinding is to identify target genes involved in this process. In an earlier study, we identified multiple potential En targets by immunoprecipitation of embryonic chromatin (Solano et al., 2003), including *eagle* and *connectin*, and also *frazzled*, *trio* and *enabled*, which are known to be involved in axonal guidance. Engrailed-mediated activation of *eagle* has been shown to play a role in the differentiation of serotonin neurons (Dittrich et al., 1997; Lundell et al., 1996). Engrailed is also known to repress the expression of the cell adhesion molecule *connectin*, which is involved in axonal fasciculation (Siegler and Jia, 1999).

Because these potential target genes are involved in the development of the VNC and are bound by En during embryogenesis, we thought that they were likely regulated by En during embryonic neurogenesis. Accordingly, we reasoned that lowering the dose of En should alter the expression of its targets, and because the interactions between En and the above-mentioned targets might be direct, this might lead to embryonic VNC phenotypes that could be observed in transheterozygous conditions. We first verified, using anti-HRP immunostaining, that embryos heterozygous for each mutation individually (*en*, *trio*, *ena* and *fra*) presented wild-type VNC architecture (Fig. 3A for en^{X31}/+, and data not shown). Genetic interactions were performed using several alleles for each mutation. *en* mutants tested corresponded either to the amorphic en^{B86} mutation or to deficiencies covering all (en^{X31}) or parts (en^{E}) of *en* and of the *en*-related gene *inv* (Gustavson et al., 1996) (Table 1).

We first tested *frazzled*, which encodes an attractive Netrin receptor that is important for axons crossing the midline (Kolodziej et al., 1996). We observed that *frazzled* is expressed

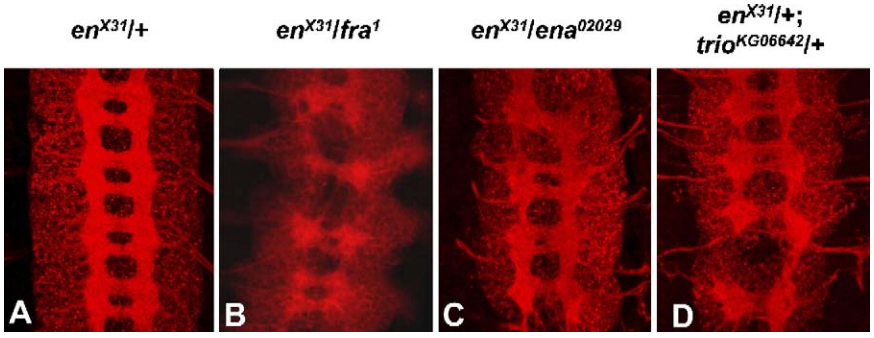

Fig. 3. *engrailed* genetically interacts with *trio*, *ena* and *fra*. VNC from stage 15 flat embryos, stained with an anti-HRP-Cy3 antibody, of the following genotypes: (A) en^{X31}/+, (B) en^{X31}/fra^{1}, (C) en^{X31}/ena^{02029}, (D) en^{X31}/+; $trio^{KG06642}$/+. VNC are oriented anterior up. All images were obtained by confocal microscopy.

in PCs and ACs (Fig. 5A), and that it is involved in the formation of both commissures, since homozygous *fra* mutants presented a strong phenotype affecting both commissures, with only a few axons ultimately crossing the midline (Fig. 5D; Ivanov et al., 2004; Kolodziej et al., 1996). Interactions between *en* and *fra* were also examined using different mutations of *fra* (fra^{1}, fra^{2}, fra^{3} and fra^{4}) (Kolodziej et al., 1996) (Fig. 3B; Table 1-A; Supplementary Fig. S1). Lethality of transheterozygous flies was scored using fra^{1} and fra^{3} mutations, whereas VNC defects were scored with the different alleles on stage 13–16 embryos, when the nerve cord is formed. In both tests (Table 1-A, C and Fig. 3B) the highest penetrance is obtained with the fra^{1} mutant, as it presented the strongest phenotype when homozygous (Fig. 5D; Ivanov et al., 2004; Kolodziej et al., 1996). In stage 15 transheterozygous en^{-}/fra^{-} embryos, using the en^{X31} deficiency, the VNC was dramatically affected, with PCs missing or fused with ACs, and longitudinal tracts thinner (see Fig. 3B as compared to en^{X31}/+ embryos in Fig. 3A, Table 1-A; Supplementary Fig. S1). Around 70% of stage 13–16 embryos presented an abnormal VNC architecture, and in these embryos nearly all the segments were affected. The positions of the *en*-expressing cells relative to the HRP staining in en^{-}/fra^{-} transheterozygous mutant embryos confirmed that PCs were particularly affected (Fig. 7F).

Table 1
Summary of genetic interactions between different *engrailed* alleles and mutations affecting target genes involved in axonal pathfinding

A. Embryonic phenotype

	fra^{1}	fra^{2}	fra^{3}	fra^{4}
en^{X31}	68% (n=73)	67% (n=85)	58% (n=147)	66% (n=103)

B. Embryonic phenotype

	en^{E}	en^{B86}	inv^{30}
fra^{1}	60% (n=145)	60.5% (n=162)	34% (n=109)

C. Adult lethality

	en^{X31}	en^{B86}	inv^{30}
fra^{1}	49% (n=257)	33% (n=258)	15% (n=470)
fra^{3}	42.1% (n=403)	ND	ND

D. Embryonic phenotype

	ena^{02029}	ena^{GC5}	$trio^{KG0664}$	$trio^{6A}$
en^{X31}	74.5% (n=106)	60% (n=130)	70% (n=110)	64% (n=127)
en^{E}	ND	15.2% (n=66)	ND	12% (n=83)

Transheterozygous combinations were analyzed and ventral nerve cord defects (A), (B) and (D) or adult lethality (C) were scored: (A) Embryonic phenotype from en^{X31} deficiency covering both *en* and *inv* and different alleles of *fra*. Phenotypes are shown on supplementary data S1; (B) Embryonic phenotype from fra^{1} and different *en/inv* mutants. Phenotypes are shown on supplementary data S2; (C) Degree of synthetic lethality from different *fra* and *en/inv* mutants; (D) Embryonic phenotype from deficiencies covering entire (en^{X31}) or parts (en^{E}) of *en* and *inv* genes, and different *trio* and *ena* mutants.
Percentages correspond to transheterozygous embryos carrying VNC defects (A, B, D) or to transheterozygous lethality (C) with n representing the number of transheterozygous embryos (A, B, D) or of flies analyzed (C).
ND: not determined. Note that in mutant embryos nearly all the segments are affected.

Different mutations affecting either *en* or *inv* were tested with the fra^{1} mutation (Table 1-B; Supplementary Fig. S2). Lethality of adult transheterozygous and axonal defects were detected in the various combinations (Table 1-B, C). In the presence of mutations that only affect *engrailed* (en^{B86}) or parts of *en* and *inv* (en^{E}), about 60% of the embryos showed axonal defects (Table 1-B; Supplementary Fig. S2), with more than 95% of the segments in these embryos affected. Finally, 34% of the transheterozygous fra^{1}/inv^{30} embryos showed similar defects (Table 1-B; Supplementary Fig. S2), with 87% of the analyzed segments in these embryos affected. Note that embryonic phenotypes show stronger penetrance in comparison to transheterozygous adult lethality, when estimated, but both show that *en* and *inv* interact with *fra*, although at a lower extent for *inv*. This confirms that both *en* and *inv* are required for VNC formation, as previously suggested (Bhat and Schedl, 1997).

In view of these results, we next used *en/inv* deficiencies to examine possible genetic interactions with mutations in other potential targets of En regulation known to be involved in axonal navigation (Fig. 3 and Table 1-D). As with *fra*, we observed that stage 15 transheterozygous en^{-}/ena^{-} embryos showed defects in CNS scaffolding with gaps in longitudinal tracts, loss of PCs, or fusion of commissures in about 70% of the segments (Fig. 3C and Table 1-D). The *enabled* gene (*ena*) encodes a regulator of the actin cytoskeleton, and is involved in axonal pathfinding in connection with cell motility and growth cone guidance (Wills et al., 1999). Ena has been shown to contribute to Robo's repulsive function on axons that do not cross the midline, and in

ena mutants, axons are less tightly fasciculated and commissures are thinner (Gertler et al., 1995; Bashaw et al., 2000).

Stage 15 transheterozygous *en*−/+; *trio*−/+ embryos showed VNC defects that were very similar to those obtained with *ena*, affecting about 75% of the segments (Fig. 3D and Table 1-D). The *trio* gene encodes a guanine nucleotide exchange factor (GEF) that is required for the proper formation of the embryonic VNC and for motor axon pathfinding (Bateman et al., 2000).

These data identify strong genetic interactions between *en/inv* and *ena*, *trio* and *fra*. The strength of the phenotypes and the high levels of defects in axonal pathfinding visualized in transheterozygous mutants, suggest a direct interaction of *fra*, *trio* and *ena* with *engrailed* during the formation of a correct scaffold within the VNC. This supports our previous findings that these genes are likely to be direct targets of En (Solano et al., 2003).

frazzled expression depends on engrailed during early embryonic development

We next analyzed the interactions between *engrailed* and *frazzled* in greater detail. Using *in situ* hybridization, we examined *fra* expression in genetic backgrounds where *en* expression is affected (Fig. 4). In wild-type stage 11 embryos, *fra* mRNA was detected in the embryonic brain (data not shown) and in a repeated segmental pattern along the A/P axis in the neuroectoderm (Fig. 4A) that partially overlapped with En expression (Fig. 4B, see marks). In *engrailed* mutant embryos, *fra* expression in the neuroectoderm was clearly reduced (Fig. 4C). Together with the above-described genetic interaction data, this suggested that En activates *fra* expression, meaning that NBs that express En should also express *fra*; this was indeed the case, as shown in Fig. 4D. Activation of *fra* by En was also confirmed by monitoring *fra* expression when En is ectopically expressed (data not shown). Together, these results suggest that Engrailed acts as an activator of *fra* transcription in NBs.

The Engrailed/frazzled interaction takes place during early development

Since En produces abnormal VNC architecture only when ectopically expressed during NB segregation (Fig. 2), and since *fra* already depends on En expression at stage 11 (Fig. 4), we asked whether the axonal defects of en^{-}/fra^{-} transheterozygous embryos results from *fra* misexpression at early or late stages. We first analyzed the Fra expression profile using an anti-Fra antibody. In wild-type embryos, Fra is expressed in a pattern similar to HRP immunostaining, and is only detectable at stage 13, when axons are formed (Fig. 5A, to compare to Fig. 1B). In stage 14 en^{X31} mutant embryos, the loss of axons in PCs (as visualized by HRP staining in Fig. 1G) correlates with a loss of Fra in the same commissures (Fig. 5B) when compared to wild-type (Fig. 5A). Indeed, in *en* mutant embryos, Fra expression is lower or absent in PCs, but normal in ACs (Fig. 5B), confirming that Fra expression depends on En in the formation of PCs, whereas Fra is involved in the formation of both ACs and PCs (Fig. 5D).

We then tested whether the activation of *fra* in En-expressing cells is essential for axonal pathfinding when occurring at early or late stages. For this purpose, we performed rescue experiments of the axonal defects seen in en^{X31}/fra^{1} transheterozygous mutant embryos (Fig. 5C). Specifically, the *prd*-Gal4 driver was used to overexpress Fra at early stages in the en^{X31}/fra^{1} genetic background. A rescue of the phenotype was observed in even segments, since 60% of the segments ($n=27$) showed normal commissures, in contrast to en^{X31}/fra^{1}, where nearly all the segments were affected (Fig. 5E, compare with Fig. 5C). A partial rescue of odd segments (30% of the segments

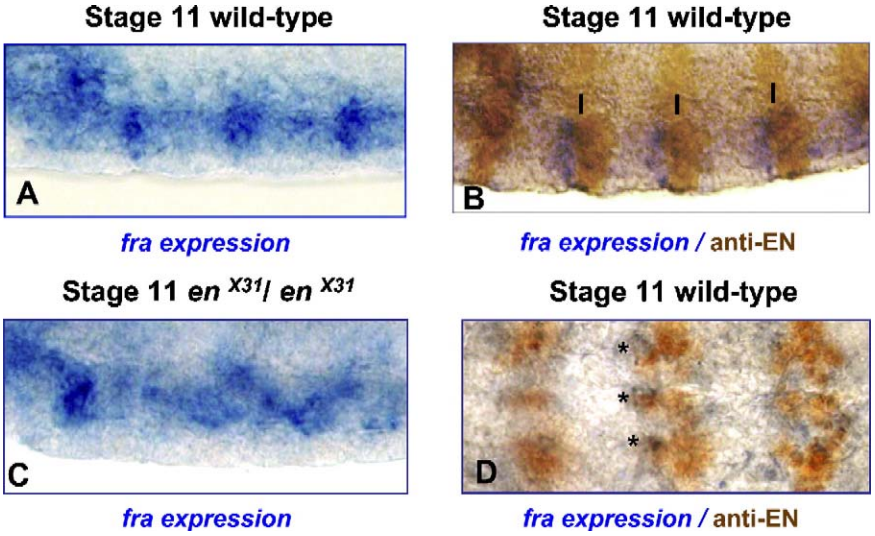

Fig. 4. *frazzled* expression depends on *engrailed* during early embryogenesis. *fra* mRNA was detected by *in situ* hybridization in stage 11 embryos (blue). (A) Lateral view of *fra* expression in wild-type stage 11 embryos. (B) Lateral view and (D) ventral view of wild-type stage 11 embryos, double-stained for *fra* mRNA by *in situ* hybridization (blue) and with anti-En by immunostaining (brown). Marks or asterisks point to cells co-expressing En and *fra*. (C) Lateral view of *fra* expression in homozygous en^{X31} embryo.

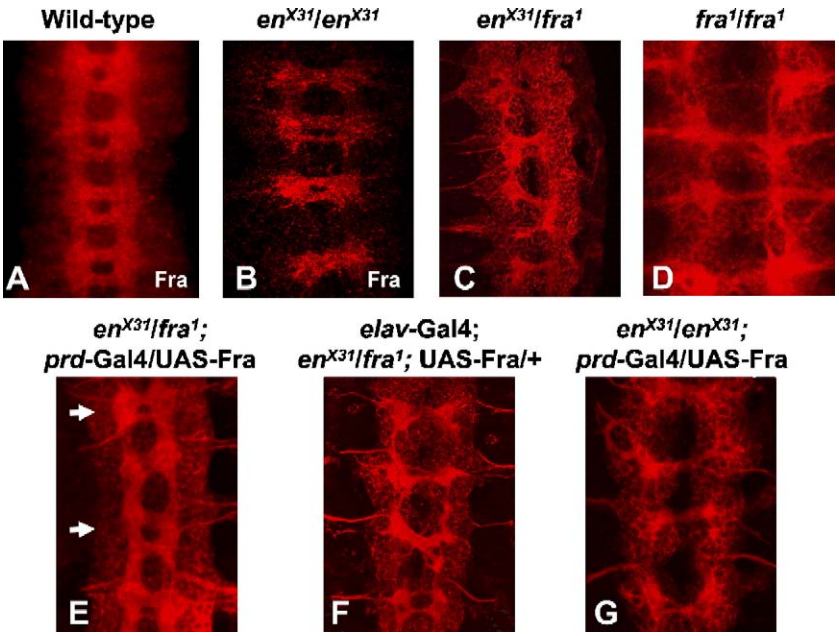

Fig. 5. Early En-regulated *fra* expression is important for axonal pathfinding. Fra expression in whole mount wild-type (A) and homozygous en^{X31} mutant (B) stage 14 embryos, as visualized by anti-Fra immunostaining. (C–G) HRP immunostaining of stage 15 embryos from following genotypes: (C) en^{X31}/fra^{1}, (D) fra^{1}/fra^{1}, (E) en^{X31}/fra^{1}; *prd*-Gal4/UAS-Fra (arrows indicate the even segments) (F) *elav*-Gal4; en^{X31}/fra^{1}; UAS-Fra/+ and (G) en^{X31}/en^{X31}; *prd*-Gal4/UAS-Fra.

($n=30$)) was also observed, probably because *prd*-positive cells also project a few axons into odd segments (data not shown). In contrast, overexpressing Fra in neurons at a later stage using the *elav*-Gal4 driver did not rescue the defects in the VNC architecture in en^{X31}/fra^{1} embryos (Fig. 5F, compare with Fig. 5C). These data suggest that the axonal growth defects observed in en^{X31}/fra^{1} embryos result from an early down-regulation of *fra* that occurs before neuronal specification.

Finally, we asked whether overexpressing *fra* using *prd*-Gal4 could rescue the abnormal pathfinding phenotype seen in en^{X31} homozygous embryos. In this case, we observed no rescue of the phenotype (Fig. 5G), which was not surprising in view of the fact that En likely regulates the expression of other genes involved in axonal pathfinding, including *eg*, *con*, *ena*, and *trio*.

The Engrailed/frazzled interaction is involved in axon growth and is important for neuronal cell fate

Since Fra has been shown to be involved in axon growth (Forsthoefel et al., 2005), and in view of the missing PC phenotype of en^{-}/fra^{-} embryos, our results suggest that *fra* activation by En during the segregation of the NBs might be necessary for the growth of axons that form PCs. To address this issue, we analyzed the behaviour of axons projecting from *eg*-expressing neurons. The zinc-finger protein Eagle (Eg) is expressed in NB 7-3 and NB 6-4, which also express En and which subsequently project axons through PCs (Dittrich et al., 1997), as well as in NB 2-4 and NB 3-3, which will project axons through ACs (Higashijima et al., 1996). Eg is expressed, for instance, in NB 7-3 just after delamination and is present in all NB 7-3 progeny until late stage 17. Therefore, in order to follow these *eg*-expressing NBs and monitor their behaviour, we analyzed *eg*-Gal4; UAS-mCD8-GFP transgenes in transheterozygous en^{X31}/fra^{1} embryos. As shown on Fig. 6, 91% of the axons that project through PCs did not grow properly ($n=21$ segments), whereas only 12% of the axonal projections ($n=17$ segments) through ACs were affected (compare Figs. 6A and B).

Since PCs are formed from En-expressing neurons (Fig. 1), we focused our analysis on those cells only. In the en^{-}/fra^{-} genetic background, we could detect that neurons still expressed En, but their position was slightly altered (Figs. 7E, F). Therefore, according to these defects in the position of neurons, we can conclude that the neuronal cell fate is affected in this genetic background. In *fra* homozygous mutant embryos, En was expressed in a striped pattern, but the position of the En-expressing neurons was also affected (Figs. 7H, I). These altered neuronal positions are likely responsible for the defects visualized in these embryos (Figs. 7D, G). We can conclude from these data that the level of Fra is crucial for neuronal cell fate and in particular for the growth of the corresponding PC axons, which were defective in both fra^{-}/fra^{-} and en^{-}/fra^{-} mutants.

Identification of an Engrailed-binding fragment within the frazzled gene

Our results suggest that the deployment of the Frazzled receptor, which is important for axon growth and for further axon pathfinding, is controlled at least in part at the transcriptional level by En. Our previous studies identified Fra as a potential direct target of En regulation (Solano et al., 2003). Indeed, immunoprecipitation of embryonic chromatin with an anti-En antibody yielded a 375-bp genomic fragment, termed 2C5, that is located within the first intron of *frazzled*

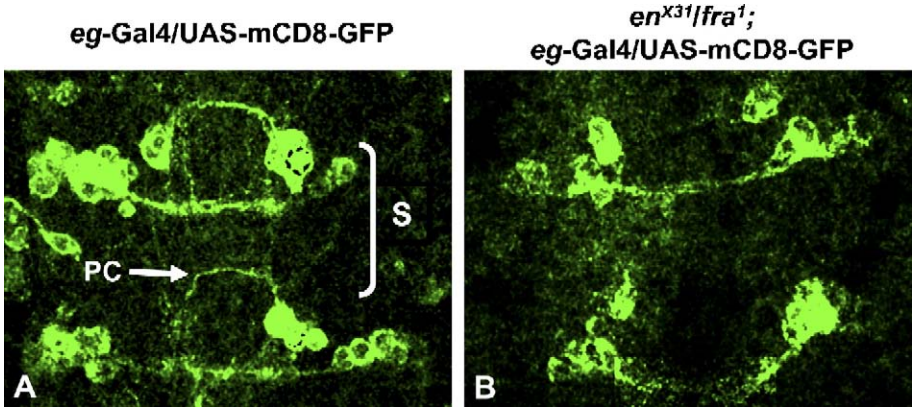

Fig. 6. Analysis of axonal projections from *eagle*-expressing neurons. (A) Stage 15 *eg*-Gal4/UAS-mCD8-GFP embryos stained by anti-GFP show cellular bodies and projections through PCs (arrow) or through ACs. S = segment. (B) Stage 15 en^{X31}/fra^{1}; *eg*-Gal4/UAS-mCD8-GFP embryos stained by anti-GFP show cellular bodies and projections through ACs, whereas projections through PCs are missing.

(Fig. 8A; Solano et al., 2003). Using gel shift assays, Engrailed was found to bind specifically to 2C5 (Solano et al., 2003). As our results were in good agreement with a role for En in activating *fra* expression, we examined whether the 2C5 fragment can recapitulate activation by En in transgenic flies carrying the fragment upstream of an mCD8-GFP reporter gene and an *hsp70* minimal promoter (Fig. 8B).

Immuno-FISH experiments on polytene chromosomes from larval salivary glands showed the presence of an additional En binding site at the transgene insertion site (Figs. 8C–F). The 2C5-GFP transgene was inserted on chromosome 2L at position 21D, as shown by *in situ* hybridization using a transgene-specific probe (Figs. 8C, D). Immunostaining with anti-En revealed an additional En binding site at position 21D (compare Fig. 8E to the control experiment with w^{1118} in Figs. 8G–I). These experiments showed that 2C5 is able to recruit Engrailed *in vivo*.

We then asked whether 2C5 can drive GFP expression *in vivo*, and, if so, whether it depends on Engrailed regulation. In the three transgenic lines that were analyzed, GFP expression was detected in third instar larval eye and wing imaginal discs (Figs. 9A and D), but not in the developing embryo (data not shown). The GFP expression pattern partially mimicked endogenous *fra* expression in these tissues (Gong et al., 1999). In the eye disc, GFP was expressed in a few photoreceptor axons, where it co-localized with the endogenous Frazzled protein (Figs. 9A–C). In wing discs, GFP was expressed in four stripes (Fig. 9D) corresponding to vein territories, coinciding in part with the endogenous Fra expression domain in this tissue (Figs. 9E, F). These data suggest that the immunoprecipitated 2C5 fragment contains a portion of the *fra* regulatory sequences.

We next tested the effect of ectopic expression of En on the 2C5-driven GFP expression pattern. For this purpose, two different forms of En were used: wild-type En, which can act as an activator or as a repressor (here referred to as En), or a chimeric construct in which the N-terminal part of the En protein has been replaced by the VP16 *trans*-activator domain (here referred to as VP16-En). This latter construct has been previously shown to always act as an activator (Alexandre and Vincent, 2003; Solano et al., 2003). Engrailed is normally expressed in the posterior territory of the wing disc (Fig. 9H) and partially co-localizes with 2C5-GFP (Figs. 9G–I). Driving VP16-En (Figs. 9J–L) or En (Figs. 9M–O) throughout the wing pouch using the *MS1096*-Gal4 driver expanded the 2C5-GFP expression domain to encompass the entire wing pouch.

Collectively, these data demonstrate that 2C5, which was identified based on its function as a binding site for En during embryogenesis (Solano et al., 2003), is also used at larval stages and may represent a portion of the *fra* regulatory sequences mediating activation by En *in vivo*. This confirms that *fra* is directly activated by En.

Discussion

During *Drosophila* development, the acquisition of NB and neuronal identity is determinant for the correct patterning of the VNC. This process is controlled in part by the combinatorial action of transcription factors (Lundgren et al., 1995; Fujioka et al., 2003; McDonald et al., 2003; Bhat and Schedl, 1997; Skeath and Thor, 2003; Maurange and Gould, 2005). The correct establishment of neuronal connectives also involves several repulsive and attractive cues that guide axons along specific pathways. While the identities of the secreted ligands, receptors, and molecular pathways involved in axon guidance are beginning to be well established (Dickson, 2002; Araujo and Tear, 2003; Kuzin et al., 2005; Forsthoefel et al., 2005), little is known about how these different factors are regulated. Perhaps the clearest example of a link between the transcriptional codes and specific guidance receptors concerns the Even-skipped (Eve) transcription factor, which was found to regulate axonal projections (Fujioka et al., 2003) through the regulation of Unc-5, a repulsive Netrin receptor (Labrador et al., 2005).

In this report, we show that Engrailed also plays a crucial role in this link, since it directly regulates actors involved in axon growth and axonal pathfinding, including the attractive Netrin receptor Frazzled.

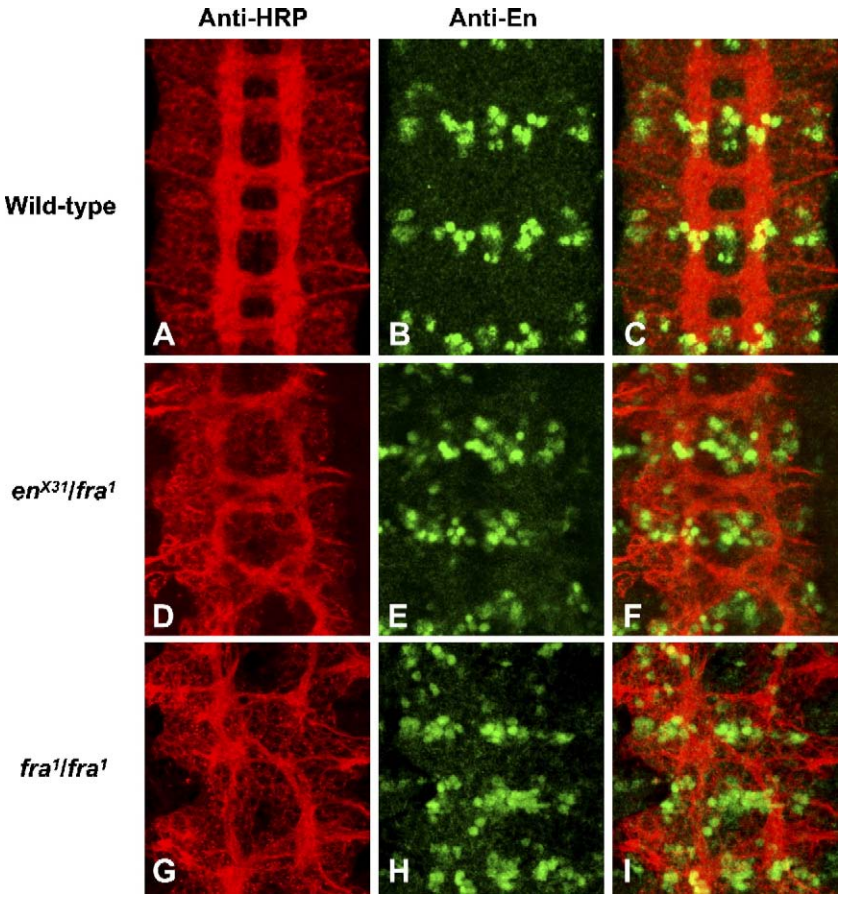

Fig. 7. Engrailed expression in en^{X31}/fra^{1} and fra^{1}/fra^{1} embryos. HRP immunostaining (in red) (A, D and G) and En immunostaining (in green) (B, E and H) in wild-type (A–C), en^{X31}/fra^{1} (D–F) and fra^{1}/fra^{1} (G–I) stage 15 embryos. (C, F and I) correspond to overlays.

Engrailed strongly interacts with different actors involved in axonal guidance

During embryogenesis, Engrailed is first expressed in posterior epidermal cells within each segment, and then later in NBs, GMCs, and neurons (Bhat and Schedl, 1997). Present at all developmental stages in a subpopulation of neural cells, Engrailed is a good candidate for a factor participating in neuronal determination (Brower, 1984; Patel et al., 1989; Lundell et al., 1996; Bhat and Schedl, 1997). We previously identified several Engrailed target genes involved in neurogenesis, and in particular in axonal guidance, including *eg*, *con*, *comm*, *fra*, *ena*, and *trio* (Solano et al., 2003). This suggested an important role for *engrailed* in this process.

Interestingly, Trio and Ena were recently found to function as effectors of Fra signalling and to act together in the formation of commissural axons (Forsthoefel et al., 2005). In particular, they were shown to physically interact, suggesting a potential mechanism by which Fra might coordinate the actin cytoskeletal dynamics necessary for axonal cone growth. We show here that *en* genetically interacts not only with *fra*, but also with *ena* and *trio*, to form the posterior commissures. En thus appears to directly regulate PC formation by acting at different levels to ensure axon growth through a complex signalling network that involves Fra.

We observed that transheterozygous embryos with alterations in both *en* and in any of several potential targets present axonal defects that are very similar to those observed in homozygous *en* mutant embryos. We also found that overexpressing Fra using the *prd*-Gal4 driver cannot rescue the axonal defects of homozygous *en* mutant embryos. This confirms that En plays an important role in axonal guidance by regulating various target genes, including *ena*, *trio*, *commissureless* (*comm*), and transcription factors such as *eg*, that have been identified as potential En targets (Solano et al., 2003; Dittrich et al., 1997). While En is often identified as a repressor, there is no evidence for a role for En in the repression of genes that instruct neurons to choose the AC, such as Wnt5/Drl components (Bonkowsky et al., 1999; Schnorrer and Dickson, 2004). Here, we demonstrate instead that En regulates axonal guidance and growth by activating components necessary for the establishment of neuronal posterior connectives.

Engrailed directly regulates frazzled expression in the embryonic ventral nerve cord

We provide here several lines of evidence that *fra* expression is directly controlled by Engrailed. For example, genomic fragment 2C5 was found to bind En *in vivo*, first during

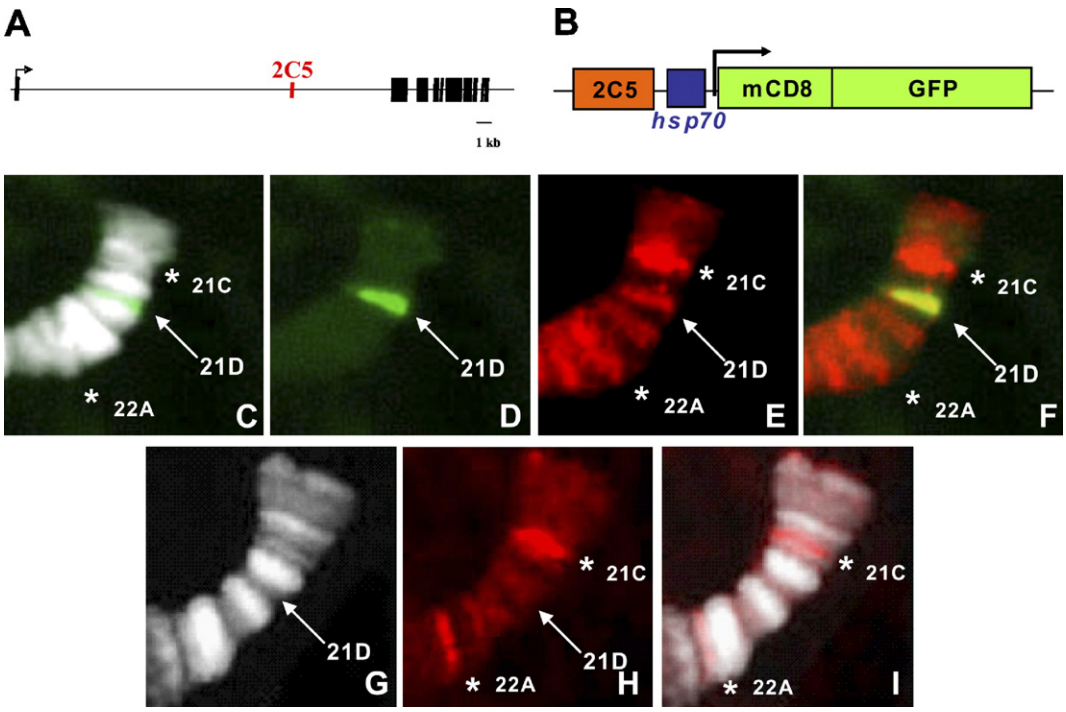

Fig. 8. Engrailed binds 2C5 on polytene chromosomes. (A) Schematic representation of the *frazzled* genomic region, highlighting in red the 2C5 immunoprecipitated fragment within the first intron. (B) Representation of the 2C5-mCD8-GFP reporter vector, in which 2C5 is inserted upstream of the *hsp70* minimal promoter and the mCD8-GFP reporter. (C–F) Polytene chromosome immuno-FISH experiments performed on the 2C5-GFP/+; ttk^{804}/+ line: (C) shows DAPI staining and *in situ* hybridization with a fluorescent probe corresponding to the transgenic construct shown in panel D, (E) anti-En immunostaining, (F) overlay of panels D and E. (G–I) Polytene chromosome immunostaining performed on the w^{1118}; ttk^{804}/+ line: (G) shows DAPI staining, (H) anti-En immunostaining, (I) overlay of panels G and H.

embryogenesis (as assayed by ChIP) (Solano et al., 2003) and later in larvae (as assayed by immuno-FISH, Fig. 9). In addition, this genomic fragment is shown here to be able to mediate activation by En in transgenic flies. However, even though it is known to bind En in embryos, 2C5 is not able to drive GFP expression during embryogenesis (data not shown), suggesting that it only recapitulates a fraction of the *frazzled* regulatory sequences.

We also provide genetic data arguing that *fra* is regulated by En during embryogenesis. *en* and *fra* interact genetically to ensure the formation of a correct scaffold within the VNC. In homozygous *en* mutant embryos, *fra* expression is affected by early stage 11, and Fra immunostaining is absent in the PCs at stage 14, correlating with a loss of posterior commissures.

The formation of the VNC depends on Engrailed regulation during NB segregation

Here, we show that PC formation requires an early function of En that acts prior to the specification of neuronal cell fate and to axon growth. Indeed, only the ectopic expression of En at early stages leads to axonal misrouting, whereas the use of pan-neuronal drivers does not cause any axonal defects. Once neurons are specified, En is no longer able to change their fate and hence affect their axonal navigation. This confirms a role for En during NB segregation (Brower, 1984; Patel et al., 1989; Bhat and Schedl, 1997; Lundell et al., 1996), and suggests that the neuronal expression of Engrailed is not essential for the formation of the VNC.

During NB segregation, Engrailed may participate in the specification of pioneer neurons. Indeed, we have observed that not all the axons that form PCs come from *en*-expressing cells. Moreover, in homozygous mutant *en* embryos, we found that the pioneer marker BP102 was affected in PCs (data not shown). This suggests that a cluster of En-positive neurons corresponds to the pioneers, which are normally required for normal pathfinding by later outgrowing neurons (Hidalgo and Brand, 1997; Sanchez-Soriano and Prokop, 2005). This could explain the absence of PCs in *engrailed* homozygous mutant embryos. Interestingly, the use of a late *eve*-Gal4 driver to ectopically express En in aCC/RP2 pioneer neurons (McDonald et al., 2003) had no effect on axonal pathfinding (data not shown). This confirms that the En-sensitive period occurs before the specification of the neurons, including the pioneers.

The Engrailed/frazzled interaction is essential during neuroblast segregation

We show in this report that the En/*fra* interaction is important for the formation of the PCs, since PCs are not formed in transheterozygous mutant en^{-}/fra^{-} embryos. This absence of PCs might result from a loss of axonal growth, which is known to involve Fra (Forsthoefel et al., 2005). This might also account for the PC defects that are observed in homozygous en^{-} mutant embryos.

Since the function of En in establishing the axon scaffold within the VNC is essential during NB segregation, we

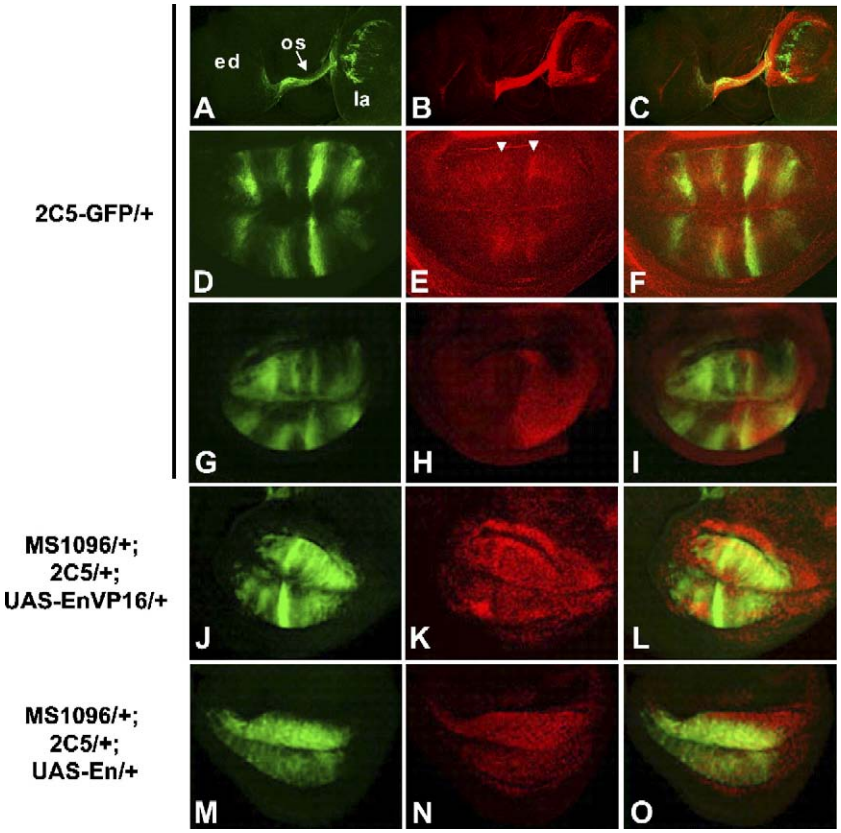

Fig. 9. Expression of 2C5-GFP line in third instar larvae. (A), (D), (G), (J) and (M) show GFP expression by anti-GFP immunostaining (green). (B) and (E) show endogenous Fra expression by anti-Fra immunostaining (red). (H), (K) and (N) show En expression by anti-En immunostaining (red). (A–C) eye disc, (D–O) wing disc. Anterior is to the left and dorsal is up. ed: eye disc, os: optic stalk, la: lamina. The genotypes are as indicated in the figure.

suspected that the regulation of En target genes involved in axonal pathfinding might also occur at early stages. Indeed, we were able to confirm that the axonal defects detected in en^{-}/fra^{-} transheterozygous embryos required the loss of early *fra* activation during NB segregation. This was shown by RNA *in situ* hybridization and by rescue experiments: PC axons of stage 15 en^{X31}/fra^{1} embryos only develop normally when Fra expression is recovered before the specification of the neurons, but do not form properly once neurons are formed. Therefore, one possible hypothesis is that the activation of *fra* in NBs allows the axonal growth of the PC pioneers.

Is the Engrailed/frazzled interaction important for neuronal cell fate?

Our data suggest that the *fra* level in NBs and neurons is crucial for axon growth. Because mutations affecting axon growth must be dominant over axonal guidance problems, it is logical that the VNCs of both en^{-}/en^{-} and en^{-}/fra^{-} present the same missing PC phenotype. Indeed, with *fra* being a direct target of En, we can assume that in the absence of the En activator, *fra* expression will be lower or lost. Indeed, we can notice that en^{-}/fra^{-} embryos phenocopy fra^{-}/fra^{-} embryos: in both cases PCs are missing, and neurons express En but show defects in their positioning. Therefore, we can attribute these changes in neuronal cell fate to a change in *fra* expression. One open question concerns the sensitive period of Fra in this process: *frazzled* is activated by Engrailed during the segregation of the NBs, but Fra protein is only detectable in neurons. One possible explanation is that Fra protein is present at early stages, but is under the threshold of detection. Another explanation can also be drawn from previous work in vertebrates, where it has been shown that growth cones possess the machinery necessary for protein translation and can translate guidance molecules locally (Campbell and Holt, 2001). The resulting rapid changes in protein levels were shown to be involved in axon guidance. Therefore, one hypothesis is that the *fra* RNA pool in NBs is rapidly translated in growth cones in order to cause changes in the cytoskeleton necessary for axon growth and their further guidance.

These results, together with previous findings, give new insights into En function during neurogenesis and show that En can alter the VNC architecture at different levels to form PCs, playing on axonal pathfinding and axon growth. Indeed, *en* mutant embryos present PCs that are not properly positioned or not even formed in most segments. Further, ectopic expression of En leads to abnormal axonal pathfinding. Both loss and gain of function of *en* could be associated with changes in the identity of the NBs (data not shown), confirming a role for En in this process (Bhat and Schedl, 1997).

En functions during neurogenesis act through the regulation of different target genes. One way is through the regulation of transcription factors such as *eagle* (Dittrich et al., 1997; Lundell et al., 1996; Solano et al., 2003), but it also regulates the expression of *fra*, *trio* and *ena*, which are more directly involved in axon growth (Forsthoefel et al., 2005) and which participate with En in the formation of the PCs. Indeed, monitoring *eg*-expressing neurons in an en^{-}/fra^{-} genetic background showed that axons projecting through PCs do not grow properly, confirming that *en* and *fra* are involved in this process.

Together, these results illustrate how En can act during NB segregation to build a wild-type VNC. Recent results in vertebrates suggest that the regulatory pathway that we have identified between En and *fra* (EN1 and DCC in vertebrates) may be evolutionarily conserved (Friedman and O'Leary, 1996; Saueressig et al., 1999; Louvi and Wassef, 2000; Ding et al., 2005). Elucidating the molecular events that allow En/Fra-positive neurons to specifically project axons through PCs but not ACs will be the next challenge to explore in order to better understand axonal guidance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.10.019.

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